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Compensatory proteolytic responses to dietary proteinase inhibitors in the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae)

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Abstract

Increasing levels of inhibitors that target cysteine and/or serine proteinases were fed to *Tribolium castaneum* larvae, and the properties of digestive proteinases were compared in vitro. Cysteine proteinases were the major digestive proteinase class in control larvae, and serine proteinase activity was minor. Dietary serine proteinase inhibitors had minimal effects on either the developmental time or proteolytic activity of *T. castaneum* larvae. However, when larvae ingested cysteine proteinase inhibitors, there was a dramatic shift from primarily cysteine proteinases to serine proteinases in the proteinase profile of the midgut. Moreover, a combination of cysteine and serine proteinase inhibitors in the diet prevented this shift from cysteine proteinase-based digestion to serine proteinase-based digestion, and there was a corresponding substantial retardation in growth. These data suggest that the synergistic inhibitory effect of a combination of cysteine and serine proteinase inhibitors in the diet of *T. castaneum* larvae on midgut proteolytic activity and beetle developmental time is achieved through the prevention of the adaptive proteolytic response to overcome the activity of either type of inhibitor.
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1. Introduction

Plant proteins have been evaluated as potential biopesticides, but many, including proteinase inhibitors, have had minimal success as insect control proteins in transgenic plants (Carlini and Grossi de Sa, 2002). Insects can respond to proteinase inhibitors in their diets by adaptation through a number of different mechanisms (reviewed in Oppert, 2000; Agrawal, 2001). Mechanisms include the stimulation of proteinase activity as well as the increased production of inhibitor-insensitive enzymes (Broadway and Duffey, 1986; Bolter and Jongsma, 1995; Jongsma et al., 1995; Broadway, 1995, 1996, 1997; Bown et al., 1997; Gatehouse et al., 1997; Overney et al., 1997; Wu et al., 1997; Mazumdar-Leighton and Broadway, 2001a,b; Cloutier et al., 1999, 2000; Rivard et al., 2004). These enzymatic responses have been documented within proteinase classes, typically with one serine proteinase replaced by another. However, in a coleopteran pest, *Callosobruchus maculatus*, a plant cystatin induced the production of aspartic (acidic) proteinase activity (Zhu-Salzman et al., 2003).

Proteinases are compartmentalized in the gut, often in regions that offer greater stability and maximal activity to the respective proteinase. In *Tenebrio molitor*, cysteine proteinases are found in the more acidic anterior midgut, while serine proteinases are found in the posterior midgut, where the region has a more alkaline pH (Thie and Houseman, 1990). Because of this compartmentalization, the shift in production of proteinases from one class to another would not be straightforward and be rather difficult for the insect to accomplish.

Abbreviations: BApNA, N-α-benzoyl-DL-arginine-*p*-nitroanilide; E-64, L-*trans*-epoxysuccinylleucylamide [4-guanidino] butane; EDTA, ethylenediamene tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SAAPFpNA, N-succinyl-ala-ala-pro-phe *p*-nitroanilide; SDS, sodium dodecyl sulfate; STI, soybean trypsin inhibitor (Kunitz).

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The larvae of red flour beetle, *Tribolium castaneum*, use primarily cysteine proteinases in food digestion (Oppert et al., 2003). In previous studies, we determined that a combination of cysteine and serine proteinase inhibitors in the diet was synergistic in inhibiting the growth of *T. castaneum* larvae (Oppert et al., 1993, 2003). One hypothesis for this synergism was that the combination of inhibitors prevented adaptive responses of the larvae to either class of inhibitors. To test this hypothesis, we fed larvae increasing amounts of a cysteine proteinase inhibitor, E-64, and a serine proteinase inhibitor, soybean trypsin inhibitor (Kunitz, STI), and evaluated the effect of these inhibitors in enzymatic assays using gut extracts in vitro. The results indicate that *T. castaneum* larvae, when fed cysteine proteinase inhibitors, compensate by shifting gut proteinase activity to serine proteinases for protein digestion. However, when cysteine and serine proteinase inhibitors are combined in the diet, the compensatory proteolytic response is prevented.

2. Materials and methods

Inhibitors used in this study included L-*trans*-epoxysuccinylleucylamide [4-guanidino] butane (E-64; Roche Applied Science, Indianapolis, IN, USA) and soybean trypsin inhibitor (Kunitz; Sigma Chemical Co., St. Louis, MO, USA). All other chemicals were obtained from Sigma, unless otherwise noted.

2.1. Insect bioassay

T. castaneum (Herbst) larvae were obtained from a laboratory culture reared continuously on 95% wheat flour mixed with 5% brewer's yeast. Prior to bioassay, larvae were placed on 85% toasted wheat germ, 10% flour, 5% brewer's yeast for optimal growth. Neonates were reared individually in 10 mg of inhibitor-treated or untreated diet per 200 μL microcentrifuge tube at 28 °C, 75% R.H. Larvae were weighed and guts were dissected when larval mass was ~1.0–1.2 mg. Mortality was recorded prior to dissection.

For dissection, larvae were first chilled on ice, the posterior and anterior ends were removed, and entire guts were excised. Guts were pooled for each treatment group in deionized water (10 guts/25 μL for proteinase assays) and frozen at −20 °C. For microplate proteinase assays, samples were thawed, vortexed briefly, and the supernatant was collected following centrifugation at 15,000×*g* for 5 min.

2.2. Zymogram analysis

Zymogram analysis with stained casein (4–16% ZBC, Invitrogen, Carlsbad, CA) was used to analyze gut proteinases from gut extracts of *T. castaneum* larvae. Proteins (0.2 gut equivalents per well) were separated by electrophoresis, and gels were incubated in renaturation buffer (1% Triton X-100 in water) for 30 min on ice, washed twice in water, followed by incubation in zymogram developing buffer, 50 mM Tris, pH 8.0, 200 mM $NaCl_2$, 0.02% Brij 35 (Invitrogen) with 5 mM L-cysteine for 4 h.

2.3. Activity blot analysis

The procedure was previously described (Oppert and Kramer, 1998). Proteins in *T. castaneum* gut extracts were separated by non-reducing sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis (PAGE) using precast 10–20% Tricine gels (Invitrogen, Carlsbad, CA). Molecular mass markers were MultiMark (Invitrogen). Proteins in electrophoresis gels were transferred to nitrocellulose and were incubated in 0.5 mg/mL N-succinyl-ala-ala-pro-phe C-nitroanilide (SAAPFpNA) in 0.1 M Tris–HCl, pH 8.1 and 0.02 M $CaCl_2$. Liberated nitroanilide was diazotized by subsequent incubations of 5 min each in 0.1% sodium nitrite in 1 M HCl, 0.5% ammonium sulfamate in 1 M HCl, and 0.05% *N*-(1-naphthyl)-ethylenediamine in 47.5% ethanol. Membranes were stored in heat-sealed bags at −20 °C.

2.4. Microplate proteinase assay

The effects of ingested proteinase inhibitors on gut proteolytic activity were evaluated in a microplate assay (Oppert et al., 1997). Maximum activity of serine proteinases occurred in pH 8.4 Universal buffer containing acetate, phosphate, and borate salts (Frugoni, 1957; Oppert et al., 2003). This buffer was selected for the microplate proteinase assay to evaluate responses to serine and cysteine proteinase inhibitors. Fluorescently labeled casein (BODIPY-TR-X casein, Molecular Probes, Eugene, OR) was diluted according to the manufacturer recommendation, and 10 μL (0.1 μg) was added to each well. Each sample was incubated in triplicate at 37 °C, and the fluorescence was measured (excitation wavelength=584 nm and emission wavelength=620 nm) and corrected by subtracting readings obtained from incubations of substrate only. Measurements of enzyme and buffer or buffer only produced negligible fluorescence and were not subtracted from readings.

3. Results

T. castaneum larvae were fed increasing amounts of E-64 or STI, and also several combinations of the two inhibitors (Table 1). Mortality ranged from 0 to 4.1% for all treatments except for the combination of 0.1% E-64 and 5% STI, for which mortality was 10%, not statistically different from the control using the Fisher's Exact Test ($p<0.05$). Survivors from each treatment were dissected when they reached weights similar to the control larvae, ensuring that larvae were actively feeding at the time of dissection. Larvae fed inhibitor treatments were older at the time of dissection,

Table 1
The effect of increasing amounts of proteinase inhibitors E-64 and/or STI in the diets of *T. castaneum* larvae on larval weight and dissection time

Treatment	Larval mass, mg (*N*)[a]	Day of dissection
Control	1.39±0.07 (53)	14
0.05% E-64	1.34±0.04 (51)	16
0.1% E-64	1.29±0.04 (55)	17
0.5% E-64	1.34±0.04 (48)	17
1.0% STI	1.39±0.05 (31)	14
5.0% STI	1.27±0.05 (27)	14
10.0% STI	1.27±0.04 (33)	15
0.1% E-64, 1% STI	1.24±0.03 (52)	26
0.1% E-64, 5% STI	1.21±0.06 (17)	29

[a] Data are mean ± S.E., *N*=number of larvae.

indicating a delay in development, as has been previously reported (Oppert et al., 1993, 2003).

Casein zymograms of extracts from larvae surviving inhibitor treatments demonstrated a specific response to either E-64 or STI (Fig. 1). Larvae fed control diets had three major caseinolytic activities (Fig. 1, lane 1). The faster migrating activity around 15 kDa was diminished as the amount of E-64 increased in the diet (Fig. 1, lanes 2–4) and was probably due to cysteine proteinase(s). An enzymatic activity around 25 kDa was stimulated as the amount of dietary E-64 increased, as well as a higher molecular mass activity greater than 60 kDa. These two proteolytic activities were diminished when STI was fed to larvae (Fig. 1, lanes 5–7) and were probably due to serine proteinases. The 25 kDa proteinase has an apparent molecular mass that is typical of many insect serine proteinases (Reeck et al., 1999). The higher molecular mass activity was observed as a "smear" during electrophoresis, which is characteristic of many lepidopteran serine proteinases that are hyperactive in the presence of SDS (Oppert, B., unpublished data). In larvae surviving a combination of 0.1% E-64 and 1% STI, the 15 and 25 kDa proteinases had reduced activity, while the >60 kDa proteinase activity was increased (Fig. 1, lane 8).

An activity blot obtained using the substrate SAAPFpNA illustrated the effect of dietary inhibitors on chymotrypsin-like activities (Fig. 2). *T. castaneum* larvae fed control diet had major chymotrypsin-like enzyme(s) with an apparent molecular mass around 20 kDa and minor enzymes of >100 kDa (Fig. 2, lane 1). These masses are similar to those of the 25 and >60 kDa caseinolytic enzymes observed in Fig. 1, and were denoted as C1–C3. Discrepancies in estimated molecular masses can be attributed to the use of different gel buffers as well as to the presence of casein in the zymogram during electrophoresis. Significant increases in activities of all chymotrypsin-like proteinases were observed as the level of E-64 increased in the diet (Fig. 2, lanes 2–4). A decrease in the activity of C1 was observed as the level of dietary STI increased, particularly at the higher dose of 10% STI (Fig. 2, lanes 5–7). The relative activity of C2 was unaffected by STI, but the activity of C3 was undetectable when larvae ingested STI. Larvae fed the combination of 0.1% E-64 and 1.0% STI appeared to have a slightly reduced level of C1 but increased levels of C2 and C3 (Fig. 2, lane 8).

The effect of 0.11 μM E-64 and 58.0 μM STI on the caseinolytic activity in gut extracts of *T. castaneum* larvae fed control (no inhibitor) and inhibitor-treated diets was examined in a microplate assay (Fig. 3). In larvae fed control diets, E-64 reduced caseinolytic activity by 87% (Fig. 3A). However, extracts from larvae fed increasing doses of E-64 were correspondingly less susceptible to E-64 inhibition. In fact, there was no inhibition of caseinolytic activity by E-64 in extracts from larvae fed the highest dose of E-64 (0.5%). In extracts from larvae fed STI, the inhibition by E-64 was not appreciably different from that of the control. In contrast, inhibition of caseinolytic activity

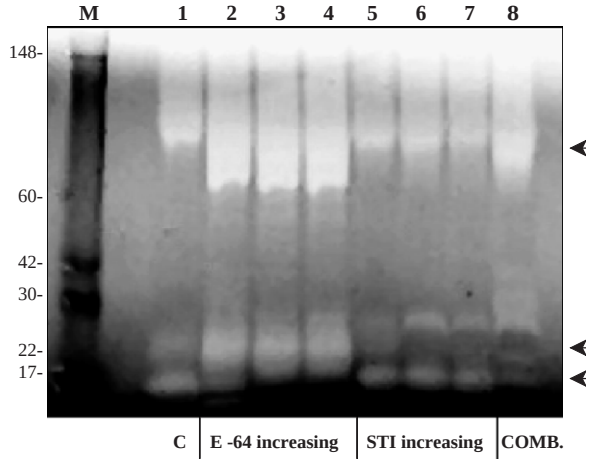

Fig. 1. Casein zymogram of gut extracts from *T. castaneum* larvae fed control (1) or diets treated with E-64: 0.05% (2), 0.1% (3), 0.5% (4); or STI: 1% (5), 5% (6), 10% (7); or a combination of 0.1% E-64 and 1% STI (8). Arrows denote those proteinases activities that are changing in response to dietary inhibitors.

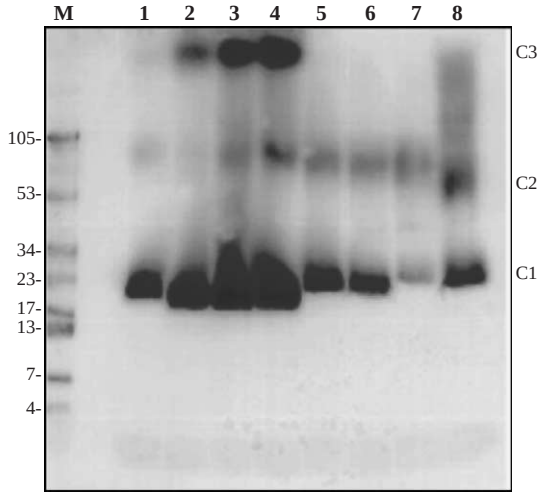

Fig. 2. Hydrolysis of SAAPFpNA by gut extracts from *T. castaneum* larvae fed control (1) or diets treated with E-64: 0.05% (2), 0.1% (3), 0.5% (4); or STI: 1% (5), 5% (6), 10% (7); or a combination of 0.1% E-64 and 1% STI (8). Chymotrypsin-like proteinase activities were denoted C1–C3, as indicated.

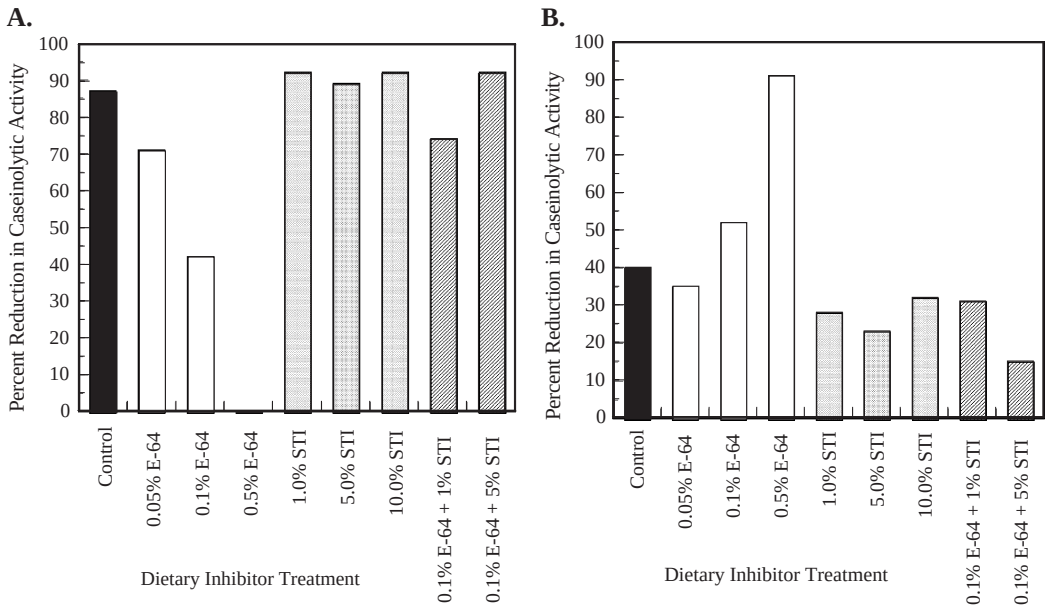

Fig. 3. The effect of (A) E-64 (0.11 μM) and (B) STI (58.0 μM) on the caseinolytic activity of gut extracts from *T. castaneum* larvae fed control or inhibitor-treated diets, as indicated.

by STI increased as the dietary level of E-64 increased (Fig. 3B). Caseinolytic activity of extracts from larvae fed control diets was inhibited 40% by STI, but inhibition in extracts from larvae fed the highest dose of E-64, 0.5%, increased to 91%. Extracts from larvae fed STI were slightly less sensitive to STI compared to the control, although there was not a consistent decline in inhibition. A combination of 0.1% E-64 and 1.0% STI in the diet resulted in a decrease in inhibition by E-64 (87 to 74%) and STI (40 to 31%; Fig. 3A and B). However, a combination of 0.1% E-64 and 5% STI led to an increase in E-64 inhibition (87 to 92%) and a decrease in STI inhibition (40 to 15%).

4. Discussion

We reported previously that a combination of serine and cysteine proteinase inhibitors was necessary to cause significant growth retardation and mortality in larvae of *T. castaneum* (Oppert et al., 1993, 2003). When either serine or cysteine proteinase inhibitors were fed individually to *T. castaneum* larvae, there were minimal effects on growth and mortality. To examine the effects of class-specific inhibitors on gut proteolytic activity, biochemical assays were used to determine the proteinase profiles in larvae fed either E-64 and/or STI. Casein zymograms indicated that larvae fed E-64 had decreased cysteine and increased serine proteinase activities, while larvae fed STI had decreased serine and increased cysteine proteinase activities. When larvae were fed a combination of inhibitors, both serine and cysteine proteinase activities in the guts were decreased. Analysis of gut extracts using a chymotrypsin-specific substrate, SAAPFpNA, revealed that larvae fed E-64 responded by increasing chymotrypsin proteinase activity, while larvae fed STI had reduced chymotrypsin activity.

It is not known whether the change in digestive proteinases is coordinated with a shift in enzyme production in different areas of the midgut. However, the shift from one enzyme class to another may have been facilitated by the excessively high quality dietary protein that provided methionine and other amino acids needed to synthesize new proteinases. Therefore, larvae feeding on lower quality diets may have more difficulty with this method of compensation.

Also of note is that another tenebrionid, *T. molitor*, has both serine and cysteine proteinases for digestion, and these activities are compartmentalized to the posterior and anterior portions of the midgut, respectively (Thie and Houseman, 1990). In larvae of *T. molitor*, the pH of the anterior midgut is 5.6, and the posterior midgut pH is 7.9. Proteinases are localized to areas where they have optimal activity. It is unknown whether this compartmentalization contributes to digestion in *T. castaneum*. However, the data from this paper suggest that such compartmentalization of enzyme activities is present in *T. castaneum* larvae as well.

In fact, conditions to evaluate the effect of proteinase inhibitors in vitro were chosen to evaluate serine proteinase activity. When conditions are altered to observe cysteine proteinases, using acidic buffers and reducing reagents, the activity of cysteine proteinases can overshadow serine proteinase activity. In previous assays under these conditions, a reduction in cysteine proteinase inhibition by E-64 was observed when larvae were fed E-64 (data unpublished). However, the effects on serine proteinases were not detectable under these conditions. Therefore, a more

alkaline buffer without reducing reagents was chosen with a general proteinase substrate to observe serine proteinase activity.

Examination of the gut proteolytic activity of larvae surviving inhibitor-containing diets provided further evidence of the compensatory response of larvae to dietary inhibitors. As the level of E-64 increased in the diets, proteinase activity was less responsive to E-64 inhibition and more responsive to STI inhibition. Less compensation was observed with STI, as might be expected because *T. castaneum* larvae are more reliant on cysteine proteinases under normal dietary conditions (Chen et al., 1992; Oppert et al., 1993, 2003). At the lower level of inhibitor combination (0.1% E-64+1% STI), a decrease in the response to either inhibitor was observed. However, at the higher combination with an increased level of serine proteinase inhibitor (0.1% E-64+5% STI), inhibition of gut extracts by E-64 increased with a corresponding decrease in STI inhibition.

These data suggest that larvae are shifting their proteolytic enzyme profile to different proteinases when fed inhibitors of either proteinase class. However, when both classes of proteinases are inhibited, larvae are unable to adapt by utilizing another class of proteinases. Therefore, an insect control strategy using a combination of inhibitors targeting multiple proteinase classes has good potential to prevent *T. castaneum* damage to stored products and cereals. Furthermore, this type of plastic enzymatic response may be common to other coleopteran as well as non-coleopteran insect pests, and a combination inhibitor approach may be effective in their control as well.

It is interesting to note that at least one other coleopteran pest, *C. maculatus*, responded to cysteine proteinase inhibitors by increasing the production of aspartic proteinases, demonstrating a shift from cysteine to aspartic proteinase classes (Zhu-Salzman et al., 2003). Larvae of *Leptinotarsa decemlineata* compensate for transgenic tomato expressing a cathepsin D inhibitor by decreasing the production of inhibitor-sensitive enzymes (Brunelle et al., 2004). In fact, this compensatory response may be more widespread, as the de novo expression of serine proteinases in an insect predator, *Perillus bioculatus*, was found in response to a diet containing transgenic potato expressing a rice cysteine proteinase inhibitor (Bouchard et al., 2003).

Plant proteinase inhibitors were once widely regarded as potential candidates for improved pest resistance in transgenic plants. However, enzymatic compensation by insects to plant proteinase inhibitors has discouraged the pursuit of new inhibitor-containing transgenic crops. The results of this study indicate that understanding enzymatic compensation to proteinase inhibitors by insects is an essential step in designing new multiple inhibitor-containing transgenic crops for insect management. Furthermore, addressing this compensation can help to improve the durability of biopesticides used for resistance to pest damage.

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